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Roles of Molecular Characteristics in Blood Anticoagulant Activity and Acute Toxicity of Sodium Cellulose Sulfate

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ABSTRACT: An attempt was made to establish correlations among molecular characteristics, blood anticoagulant activity, and acute toxicity of sodium cellulose sulfate (NaCS). For this purpose, 25 NaCS samples ranging in number-average molecular weights M_n from 800 to 36.8×10^4 and in total degree of substitutions $\langle\langle F \rangle\rangle$ from 0.5 to 2.75 were prepared. The distribution of substituent groups on three carbon positions (C_2, C_3, and C_6) in a glucopyranose unit $\langle\langle f_k \rangle\rangle$ ($k = 2$, 3, and 6) of NaCS was determined by the NMR method. The anticoagulant activity of NaCS to the whole blood was evaluated by the methods of Lee-White, Imai, and the Commentary of Japanese Pharmacopoeia. Acute toxicity, as measured by LD_{50} (vein injection), of NaCS for rats was determined by the Probit method. Inhibitory action of NaCS on blood coagulation was estimated using a coagulation factor deficient substrate plasma and a sample plasma from normal blood. It was found that the sum $\langle\langle f_2 \rangle\rangle + \langle\langle f_3 \rangle\rangle$ is a predominant factor for the anticoagulant activity of NaCS, but M_n is only a minor factor. LD_{50} of NaCS was found to be intricatedly influenced by $\langle\langle f_2 \rangle\rangle + \langle\langle f_3 \rangle\rangle$ and M_n. The inhibitory action of NaCS towards coagulation factor VIII was found to be much more intensive than heparin. An anatomical study showed that after injection of NaCS, no abnormality occurred in live rats but a state of anemia appeared in the liver of dead rats and congestive and blood shots occurred in their lungs.

KEY WORDS Sodium Cellulose Sulfate / Total Degree of Substitution / Number-Average Molecular Weight / Anticoagulant Activity / Acute Toxicity / Polymer Drug /

The chemical structure of cellulose sulfate (CS) resembles those of muco-polysaccharides, such as heparin and condroitin sulfate, which are now in wide use as natural-occurring blood anticoagulants. Figure 1 shows (a) three possible positions of the sulfate group in the glucopyranose unit of sodium cellulose sulfate (NaCS) and (b) the chemical structure of the uronic linkage in heparin. NaCS is linked together by 1,4-β-glucoside, but heparin by 1,4-α-glucoside. An anticoagulant activity of NaCS was first reported by Bergström[1] as early as 1935, and thereafter the Biological Institute of Carlsberg Fundation in Copenhagen demonstrated that the coagulation time of whole blood increased by the addition of cellulose trisulfate and other polysaccharide sulfates such as amylose sulfate and amylopectin sulfate.[2-6] Felling and Wiley, and Rothschild and Castania found many interesting pharmaceutical characteristics of NaCS including 1) inhibitory action to pancreatic ribonuclease,[7] 2) kininogen depleting action,[8] 3) endo-toxin shock of dogs by the treatment of NaCS.[9] The aminosulfate group at the C_2 position of uronic units in heparin was found to play an important role in anticoagulant activity.[10] Desulfation of this aminosulfate group was found to lower the anticoagulant activity of heparin. These experimetal results on heparin suggest strongly that the physiological

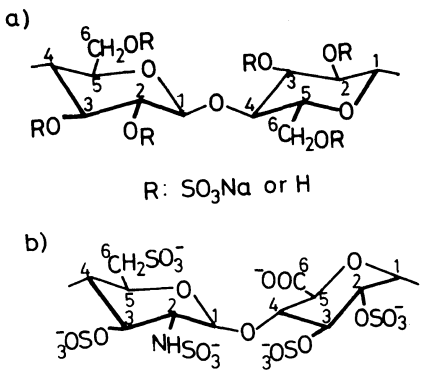

Figure 1. Chemical structure of sodium cellulose sulfate (a) and heparin (b). Here, the C1 chair conformation is assumed for both compounds.

activity, including anticoagulant activity, of polymers is closely related to their molecular characteristics. Thus, we may expect for NaCS that its anticoagulant activity and other pharmaceutical activities are influenced by molecular parameters such as molecular weight, chain structure, distribution of substituent groups along the molecular chain, the probability of substitution at C_2, C_3 and C_6 positions of glucopyranose units ($\langle\langle f_k \rangle\rangle$, $k=2$, 3 and 6), and the total degree of substitution $\langle\langle F \rangle\rangle$. Unfortunately knowledge of the molecular characteristics of NaCS is as yet in a very primitive state because of its less practical importance and the experimental difficulty associated with its polyelectrolytic nature.

We recently succeeded, by nuclear magnetic resonance (NMR), in determining the distribution of sulfate groups in glucopyranose units of NaCS[11] and also evaluated, by viscometry, membrane osmometry and light scattering, the dilute solution properties of this polymer.[12] This article attempts to find a correlation of molecular characteristics of NaCS with its anticoagulant activity and acute toxicity.

EXPERIMENTAL

Synthesis of Sodium Cellulose Sulfate (NaCS)

25 samples of sodium cellulose sulfate (NaCS) having number-average molecular weight $M_n=800$—36.8×10^4 and total degree of substituent $\langle\langle F \rangle\rangle=0.50$—2.75 were synthesized as follows: 20 gram (g) of acid hydrolyzed wood pulp (viscosity-average degree of polymerization = 50—3000) in the form of sheet or flock was allowed to absorb 40 ml of dimethylformamide (DMF). The DMF-wetted pulp thus prepared was mixed at room temperature with a given amount (86—130 g) of DMF/sulfur trioxide (SO_3) complex (2 : 1, mol/mol) precooled at 0°C. The mixture was stirred vigorously at 25°C for 4 h. The resultant solution was diluted with a large volume of water, immediately neutralized with 2*N* sodium hydroxide (NaOH), and methanol was added to it to precipitate the crude NaCS. The dried crude NaCS was dissolved in distilled water and the resulting brownish solution was passed through a diatomaceous earth layer to remove any impurities, and the polymer was reprecipitated with methanol. The polymer was washed several times with a mixture of methanol/water (7 : 3, v/v) and again dissolved in deionized water and finally subjected to dialysis against deionized water until the electroconductivity of the dialyzing water became below 10^{-5} MS/cm. Pure NaCS obtained by the evaporation of the solvent was designated as CS. By this method, 16 NaCS samples with $\langle\langle F \rangle\rangle=1.36$—2.75 were prepared. Of these samples, CS-11 was resulfated under the same conditions used above and designated CSD. This had $\langle\langle F \rangle\rangle=1.99$, which was almost the same as that of CS-11, but had a different $\langle\langle f_k \rangle\rangle$. For example, $\langle\langle f_6 \rangle\rangle$ changed from 0.3 to 0.67 by resulfation (see Table I). In order to lower $\langle\langle F \rangle\rangle$, the above synthetic procedure was modified so that the solution of NaCS in DMF/SO_3 was heated at 70°C for 30 min before neutralization. This procedure gave NaCS having $\langle\langle F \rangle\rangle=1.05$, and the product was coded as DSH. A NaCS sample coded as CS-A was obtained from a pulp depolymerized by repeated acid- and alkali-hydrolysis, using the same conditions as in preparing CS samples. NaCS with low $\langle\langle F \rangle\rangle$ was prepared by the following procedure: Acid-hydrolyzed pulp was immersed in a large volume of a mixture of 98% sulfuric acid (H_2SO_4)/*n*-butanol (110 : 29, v/v) at 5—10°C for 10 min and the excess mixture was squeezed out of the pulp by hands. The resulting gelatineous liquid was maintained at 0—5°C, then partially neutralized with 30% aqueous NaOH, and crude CS was precipitated by adding diethylether containing dry carbon dioxide. The viscous crude CS was again neutralized with 5% aqueous NaOH and precipitated with methanol. The precipitated NaCS was purified by the same method as in the DMF/SO_3 complex

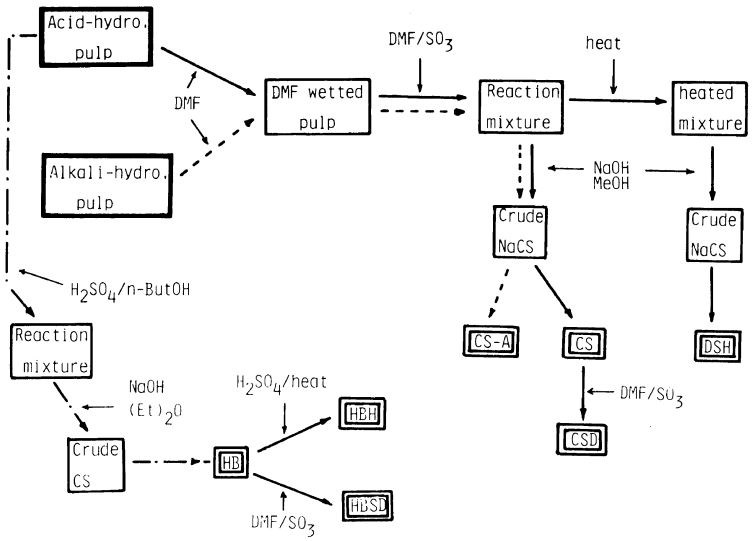

Figure 2. Schematic representation of the synthesis procedures of NaCS. Double rectangles indicate sample codes (see the text).

method, and coded HB. Four HB samples obtained had ⟪F⟫ of 0.7—0.95. NaCS coded as HBSD was obtained by resulfating sample HB-1 using the DMF/SO_3 complex. ⟪F⟫ of HBSD was the same as that for HB-1 (⟪F⟫ = 0.95), but the ⟪f_k⟫ was different. NaCS with extremely low M_n, coded as HBH, was obtained by heating sample HB-1 in 6N aqueous H_2SO_4 at 70°C for 1 h. ⟪F⟫ of HBH was 0.5, lower than that of HB-1. All synthetic procedures used in this study are summerized in Figure 2.

A commercial sample of heparin sodium salt, which had an anticoagulant activity χ of 152 IU/mg as measured by Japanese Pharmacopoeia, was obtained from Nakarai Chemicals Co. Ltd. (Kyoto). A purified heparin with χ of 188—228 was prepared by treating this commercial heparin with ethanol.

Molecular Weight of NaCS

The M_n of the NaCS samples were determined with a Melab membrane osmometer model CMS-1 and a Hewlett-Packard high-speed membrane osmometer model 502 at 25°C with 0.5M aqueous NaCl as a solvent.[12] Selecton B 19 (made by Schleiche & Schnell Inc.) was used as the membrane.

⟪F⟫ and the Distribution of Sulfate Groups in Glucopyranose Units

The total degree of substitution, ⟪F⟫ weight-averaged over all glucopyranose units, was determined gravimetrically according to the method described by Schweiger[13] and denoted as $\langle\langle F \rangle\rangle_g$. The amount of sulfric acid liberated by boiling NaCS in 1*N* hydrochloric acid was taken as that of barium sulfate. The glucopyranose unit constituting a cellulose molecule has one primary hydroxyl group at C_6 and two secondary hydroxyl groups at C_2 and C_3 (see Figure 1). The degree of sulfate substitution is different at different C positions, but its average over the three C positions is essential for characterizing NaCS. We denoted the average probability of hydroxyl group substitution at the C_k ($k = 2$, 3 and 6) position by ⟪f_k⟫ and determined it for 13 NaCS samples, using the ^1H NMR method proposed by Kamide and Okajima.[11] The sum of ⟪f_2⟫, ⟪f_3⟫ and ⟪f_6⟫ is denoted by $\langle\langle F \rangle\rangle_{NMR}$.

Evaluation of the Anticoagulant Activity of NaCS

Method of Lee-White. Anticoagulant activity (A_{LW}) was evaluated by a modification of the method of Lee-White[14] in a vessel thermostated at 37°C. The modification was made as follows. The inner wall of a polyethylene injector (inner diameter 1.0 cm, volume 2.5 ml, manufactured by Nippro

Co.) was first washed with physiological saline. 1.0 ml of physiological saline dissolving an anticoagulant at a concentration of 0.1 wt% was sucked into the injector and the anticoagulant solution was pressed out by hand. It was expected that a trace of anticoagulant remained adhering to the inner wall of the injector. 0.1 ml of fresh human whole blood was admitted into the injector and 0.1 ml of air was sucked into the injector. During this operation, the injector was maintained in an upright position. We measured the time t_1 necessary for the test blood to start forming small coagulated blood gel particles by observing the blood flow pattern on the injector wall while the injector was gently being shaken. We also measured the time t_2 required for completion of blood coagulation (*i.e.*, clotting or thrombi), as usually done in measuring anticoagulant activity. These two times t_1 and t_2 were found to be closely correlated to each other. After the completely coagulated blood gel was removed by placing the injector upside down, the amount of blood m_1 remaining on the wall was roughly estimated. By comparing the results with those of heparin, the anticoagulant activity A_{LW} of NaCS estimated by the Lee-White method was classified into four grades: superior (S), good (G), inferior (I), and non-active (N).

Method of Imai[15]. The amount of formaldehyde-fixed thromb was determined at 37°C. A watch glass of 11 cm in diameter was first washed with physiological saline, wiped with pure cotton gauze, and then 0.02 ml of physiological saline containing 1.0 wt% anticoagulant was dropped onto the center of the watch glass and spread by swirling action. 0.30 ml of the fresh human whole blood was placed on this glass, made to stand for a given time, fixed with 37% formaldehyde, washed with water, dried in air, and the weight of thromb formed, m_2, was measured with a precision balance.

Method According to the Commentary of Japanese Pharmacopoeia. The method described in the Commentary of Japanese Pharmacopoeia[16] provides another parameter of anticoagulant activity, χ, which is expressed in international units (IU)/mg of heparin. For the evaluation of χ for NaCS, the original procedure was slightly modified as follows:

(1) Prepare solutions of different concentrations by dissolving a heparin standard with χ of 152 IU/mg (hereafter denoted as χ_0) or NaCS in physiological saline.

(2) Add 0.02 ml of these solutions to 1.0 ml of the fresh human whole blood (B^-, male adult) and measure the coagulation time (t_3) in second. Measurements are made at different concentrations ($0—75 \times 10^{-3}$ mg ml^{-1}) of the heparin standard and the polymer sample.

(3) For each solution, plot $\log t_3$ against the absolute amount m_3 (in mg) of the anticoagulant used.

(4) Determine the slopes of the plots for both the standard and the polymer sample.

(5) Calculate χ (IU/mg) of the polymer sample from

$$\chi = \chi_0 \times \frac{\mathrm{d} \log t_3 / \mathrm{d}m_3}{(\mathrm{d} \log t_3 / \mathrm{d}m_3)_0} \qquad (1)$$

where the subscript o denotes the heparin standard. The plot in step (4) was found to give a straight line.

Physiological Action of NaCS to Various Coagulation Factors in Blood

All assays of the physiological action of NaCS to coagulation factors were made at 37°C with heparin as the reference. We used specially treated plasma, in which one of the coagulation factors was deficient

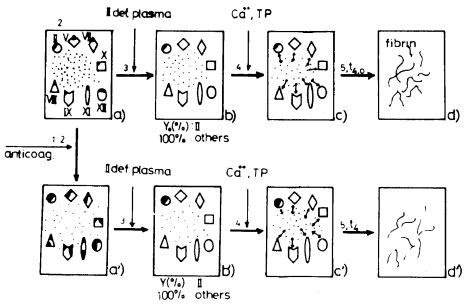

Figure 3. Scheme of the principle for measuring the existence ratio Y of coagulation factor II: Numbers on the figure denote the steps described in the text.
(a) sample plasma from normal human whole blood. Shaded area indicates $(100 - Y_0)$ (%).
(b) sample plasma containing a coagulation factor II deficient substrate plasma. Y of all factors, other than that of factor II gives 100% when added.
(c) Fibrinogen in (b) was activated by addition of activated thromboplastin.
(d) Coagulated plasma in which fibrin was formed.
(a′) sample plasma containing anticoagulant NaCS. Shaded area indicates $(100 - Y_0)$ % and black area $(Y_0 - Y)$ %.
(b′)—(d′) equals (b)—(d); dots denote fibrinogen.

(hereafter referred to as a coagulation factor deficient substrate plasma) and evaluated the percentage of the coagulation factors existing in a given sample plasma. For this purpose, the conventional procedure was modified by adding in advance an anticoagulant to the sample plasma. The procedure scheme is given in Figure 3. The details are as follows:

a) *Determination of the Existence Ratios Y of Internal Coagulation Factors II, V, VII, and X.*

(1) Put 0.01 ml of physiological saline solution containing 2×10^{-4} mg of heparin or 1.6×10^{-4} mg of NaCS in a test tube having an inner diameter of 0.8 cm and maintained at 37°C.

(2) Dilute one volume of the sample plasma with nine volumes of an Olenveronal buffer solution, supplied by Diagnostic Inc., USA, and put 0.1 ml of this solution in the above-mentioned tube and leave the system at 37°C.

(3) Prepare four kinds of coagulation factor deficient plasmas (deficient in II, V, VII, and X, respectively) (Diagnostic Inc., USA). Add 0.1 ml of the plasma to 0.1 ml of the solution in step (1) and allow the tube to stand for 2 min.

(4) Blow 0.1 ml of an activated thrombo-plastin/calcium chloride mixture (Diagnostic Inc., USA) into the tube in step (3).

(5) Measure the time (t_4) necessary for fibrin to start growing.

(6) Calculate the existence ratios Y (%) of individual coagulation factors using the following equations, derived from the experimental relationships supplied in the form of graphs by Diagnostic Inc., USA.[17]

$$\log t_4 = -0.19 \log Y + 1.475 \quad \text{for factor II} \tag{2}$$

$$\log t_4 = -0.30 \log Y + 1.879 \quad \text{for factor V} \tag{3}$$

$$\log t_4 = -1.445 \log Y + 1.742 \quad \text{for factor VII} \tag{4}$$

$$\log t_4 = -0.2824 \log Y + 1.799 \quad \text{for factor X} \tag{5}$$

b) *Determination of the Existence Ratios Y of External Coagulation Factors VIII, IX, XI, and XII.* For this purpose, we slightly modified the steps for the determination of internal coagulation factors as follows. In step (2), one volume of the sample plasma was diluted with four volumes of the Olenveronal buffer, and in step (4), a cepharoplastin/calcium chloride mixture was used in place of the thromboplastin/calcium chloride mixture. The values of Y of the external coagulation factors were calculated from the experimental relations:

$$\log t_4 = -0.1709 \log Y + 2.0325 \quad \text{for factor VII} \tag{6}$$

$$\log t_4 = -0.1776 \log Y + 2.0363 \quad \text{for factor IX} \tag{7}$$

$$\log t_4 = -0.250 \log Y + 2.1236 \quad \text{for factor XI} \tag{8}$$

$$\log t_4 = -0.2189 \log Y + 2.22147 \quad \text{for factor XII} \tag{9}$$

In the procedures described in a) and b), some loss may occur in the activity of the coagulation factors when the sample plasma and the anticoagulant are mixed. However, it is assumed here that when a coagulation factor deficient substrate plasma is added to the mixture of normal plasma and an anticoagulant the activities of all coagulation factors other than the deficient coagulation factor recovered to 100%. The fundamental concept underlying the method for determining Y is illustrated in Figure 3. Since the values of Y for various coagulation factors in the sample plasma were determined after adding the anticoagulant, the ability of an anticoagulant to inactivate the coagulation factor under consideration can be defined by $100(Y_0 - Y)/Y_0$, where Y_0 is the value of Y when no anticoagulant is added. It should be noted that here Y was obtained only at one dose level (2×10^{-4} mg). Linear relations between the logarithmic coagulation time t_4 (expressed in second) and the absolute amount m_4 of anticoagulant were confirmed at least within the concentration range of the anticoagulant studied. Thus, the slope of the plot of $\log t_4$ *vs.* m_4 could be taken as another measure of the inhibitory action of the anticoagulant toward the coagulation factor.

LD_{50} When Anticoagulant is Injected Into the Vein of a Rat

Four-weeks-old male rats weighing 21—23 g were used. The rats were confined to shaving beds in a plastic cage and allowed to eat and drink at will. The cage was changed every 3—4 days. An NaCS solution (concentration 0.25—0.8 wt%) in physiological saline was injected into a vein at a rate of 0.1 ml/10 s, and general symptoms and changes in body-weight were observed during the following three weeks, using 10—20 rats for a given dose level. LD_{50} was determined from the death ratio one week after the injection, according to the Canola-SX-50 (Probit) method.[18] Both dead and live rats during the test were subjected to anatomy and intestinal abnormalities were visually examined.

RESULTS AND DISCUSSION

Figure 4 shows some typical ^{1}H NMR spectra of NaCS in deuterium oxide. Figures 4a) and b) refer to two NaCS samples having nearly the same ⟪F⟫ (1.96—1.97), but different ⟪f_k⟫. ⟪F⟫ decreased in the order: a)≃b)>c)>d). The NMR signal for the proton at the C_1 position shifts very slightly to higher magnetic field as ⟪F⟫ decreases. The structure of the spectrum in the range from 3.4 to 4.0 ppm becomes complicated with a decrease in ⟪F⟫, approaching that for pure cellulose.

Table I lists the values of M_n, ⟪F⟫, and the anticoagulant parameters A_{LW}, m_2 and χ, and LD_{50} of 25 NaCS samples and heparin. For the selected 13 NaCS samples, the distribution of sulfate groups over the three possible positions in a glucopyranose unit (⟪f_k⟫ ($k=2$, 3 and 6)) is also given in this table. It can be seen that $\langle\langle F\rangle\rangle_{NMR}$ agrees with $\langle\langle F\rangle\rangle_g$ within an uncertainty of ± 0.10. The correlation coefficient between these was estimated to be 0.998. In a previous study[12] it was found that the ratio of weight- to number-average molecular weight M_w/M_n of CS samples ranged from 3.1 to 4.1. The procedures employed here for synthesis of NaCS afforded samples having a great variety of M_n and ⟪F⟫. Thus, the M_n of these sample varied from 800 to 36.8×10^4 and their ⟪F⟫ from 0.5 to 2.75.

Figure 5 shows the relation between A_{LW} and m_2 or χ. A_{LW} of the S grade corresponds to m_2 of 0.4—2.8 and χ of 188—246 and A_{LW} of the grade G to m_2 of 4.8—5.3 and χ of 152—206. A_{LW} of the I grade ranges in m_2 from 12.6 to 26.3 and in χ from 58 to 124 and A_{LW} of the N grade corresponds to m_2 larger than 42 and χ lower than 15. Some overlaps and gaps in m_2 and χ for different grades exist because of the qualitative nature of A_{LW} and also because of the insufficient number of polymer samples. It is of interest to see that NaCS has a wide range of anticoagulant activity.

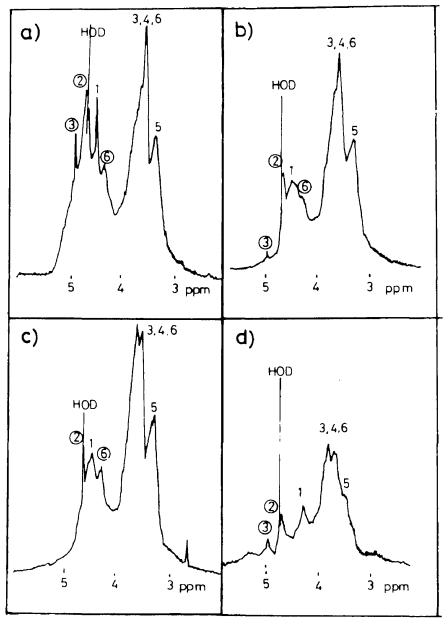

Figure 4. Typical ^{1}H NMR spectra of NaCS samples in deuterium oxide: a) CS-A; b) CS-11; c) HBSD; d) HBH. Numbers indicate the position of carbon atoms (see Figure 1) and those in the circles denote the position of carbon atoms linking the hydroxyl residues.

Figure 6 shows the relation between A_{LW} and ⟪F⟫ of NaCS. Qualitatively, A_{LW} has a tendency to approach the G or S grade with an increase in ⟪F⟫. In particular, for samples with ⟪F⟫ less than 1.94, A_{LW} was of neither G nor S grade, and all HB samples which had ⟪F⟫ less than unity, showed very low anticoagulant activity. ⟪F⟫ is not the only factor controlling A_{LW}, because when ⟪F⟫ is in the range 1.88—2.05, A_{LW} covers all grades varying from the N to the S. All CS samples having ⟪F⟫ larger than 1.88 showed A_{LW} of the S or G grade, but sample CS-A, which had ⟪F⟫ of 1.97, showed A_{LW} of the N grade. It should be noted that there is a great difference in ⟪f_2⟫+⟪f_3⟫ between the CS samples (>1.6) and sample CS-A (0.97). Sample CSD had almost the same ⟪F⟫ (=2.00) as sample

Table I. Characterization of sodium cellulose sulfate

Sample	$M_n \times 10^{-4}$	Total ⟪F⟫		Distribution of sulfate			Anticoagulant activity			LD_{50}
		Gravimetry	NMR	⟪f_2⟫	⟪f_3⟫	⟪f_6⟫	A_{WL}	m_2/mg	χ/IU mg^{-1}	mg kg^{-1}-rat
CS-1	36.8	1.66	—	—	—	—	I[a]	26.3	—	—
CS-2	9.39	1.94	—	—	—	—	G[b]	—	—	—
DSH	6.91	1.05	1.05	0.55	0.00	0.50	N[c]	—	0	730
CS-3	6.79	1.98	—	—	—	—	G	—	—	—
CS-4	6.65	2.39	2.46	1.00	0.74	0.72	G	4.8	184—228	53.6
CS-5	5.32	1.36	1.36	0.75	0.42	0.19	I	—	55—61	513
CS-6	5.31	1.84	—	—	—	—	I	12.6	—	—
CS-7	5.06	1.84	—	—	—	—	I	—	—	—
CS-8	5.06	1.73	—	—	—	—	I	—	—	—
CS-9	4.98	1.81	—	—	—	—	I	—	—	—
CS-10	4.32	2.75	2.75	1.00	0.92	0.83	S[d]	0.8	230—242	25.2
CS-A	3.15	1.97	1.97	0.71	0.26	1.00	N	42.0	7—8	5000
CS-11	2.48	1.98	1.96	1.00	0.61	0.34	S	2.8	175—219	43
HB-1	2.26	0.95	0.95	0.52	0.00	0.43	N	46.3	0	4634—5326
CS-12	1.95	2.03	1.93	1.00	0.65	0.28	S	1.2	200—222	63.8
CS-13	1.80	1.74	—	—	—	—	I	—	—	—
CS-14	1.70	2.60	2.62	1.00	0.87	0.85	S	0.4	242—251	38.3
CSD	1.69	2.05	1.99	0.75	0.55	0.67	I	—	117—130	726
CS-15	1.57	1.96	—	—	—	—	S	0.6	—	—
CS-16	1.57	1.94	1.97	1.00	0.60	0.37	S	0.6	167—209	184.5
HB-2	1.24	0.70	—	—	—	—	I	18.5	—	—
HB-3	1.16	0.74	—	—	—	—	I	13.7	—	—
HBSD	0.99	0.95	0.95	0.29	0.33	0.33	N	—	10—11	1004
HB-4	0.92	0.76	—	—	—	—	I	12.7	—	—
HBH	0.08	0.50	0.58	0.31	0.27	0.00	N	—	12—15	712
Heparin	—	—	—	—	—	—	G	5.3	152	1200

[a] I denotes inferior.
[b] G denotes good equivalent.
[c] N denotes non-active.
[d] S denotes superior.

CS-11. The most significant difference between these two polymers is that CSD had a lower ⟪f_2⟫+⟪f_3⟫ (or ⟪f_2⟫ and ⟪f_3⟫), and its A_{LW} was one grade below that of CS-11. Attempts to increase ⟪F⟫ by resulfation of sample HB-1 with the DMF/SO_3 complex failed, and no improvement of A_{LW} was obtained in spite of the resulting marked changes in ⟪f_k⟫ (for example, ⟪f_3⟫ changed from zero to 0.34). These facts suggest that A_{LW} is related not only to ⟪F⟫ but also to the average distribution of the substituent groups in glucopyranose units (⟪f_k⟫, $k=2$, 3 and 6).

Figure 7 shows A_{LW} of NaCS as a function of ⟪f_2⟫, ⟪f_3⟫, ⟪f_6⟫, and ⟪f_2⟫+⟪f_3⟫. ⟪f_2⟫, ⟪f_3⟫, and ⟪f_2⟫+⟪f_3⟫ show good correlation with A_{LW}, but ⟪f_6⟫ does not. All NaCS samples with ⟪f_2⟫<0.75 or ⟪f_3⟫<0.48 have A_{LW} of the N grade (in this case, ⟪f_2⟫ and ⟪f_3⟫ are closely correlated with one another). ⟪f_2⟫+⟪f_3⟫ has the best correlation with A_{LW}.

Table I suggests that the average molecular weight M_n has a significant effect on A_{LW}. As far as CS samples are concerned, A_{LW} tends to increase with a decrease in M_n.

Figure 8 shows the relation between χ and the distribution of the sulfate groups, represented by (⟪f_2⟫+⟪f_3⟫)/3, ⟪f_6⟫/3, and (3−⟪F⟫)/3. The theoretical maxima of (⟪f_2⟫+⟪f_3⟫)/3, ⟪f_6⟫/3, and ⟪F⟫ are 0.67, 0.33, and 3, respectively. The full lines in the figure indicate the contours of the same χ. χ is

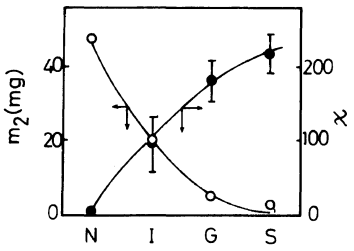

Figure 5. Correlation between anticoagulant activity A_{LW}, measured by the Lee-White method, and weight of thromb m_2 determined by the Imai method, or anticoagulant activity χ (IU/mg) measured according to the Commentary of Japanese Pharmacopoeia.

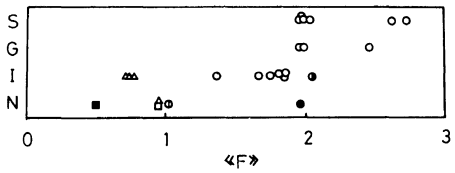

Figure 6. Effect of total degree of substitution ⟪F⟫ of NaCS on anticoagulant activity A_{LW}: ○, CS series; ⊙, CSD; ◑, DSH; △, HB series; ●, CS-A; □, HBSD; □, HBH.

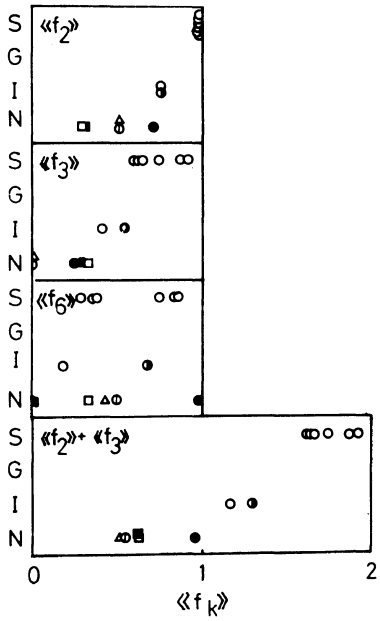

Figure 7. Effect of ⟪f_2⟫, ⟪f_3⟫, ⟪f_6⟫ and ⟪f_2⟫+⟪f_3⟫ of NaCS on anticoagulant activity A_{LW}. Symbols have the same meaning as those in Figure 6.

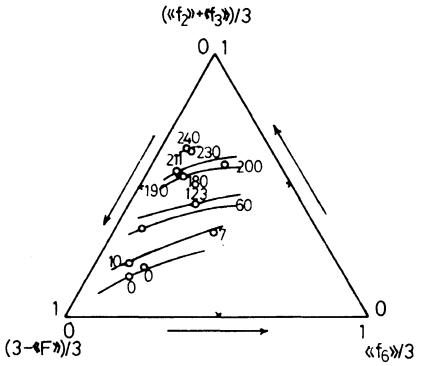

Figure 8. Dependence of anticoagulant activity χ of NaCS on (⟪f_2⟫+⟪f_3⟫)/3, ⟪f_6⟫/3 and (3−⟪F⟫)/3: ⟪f_k⟫ is the distribution of sulfate groups on the carbon position k in glucopyranose units. χ is given on each curve. Open marks, experimental data.

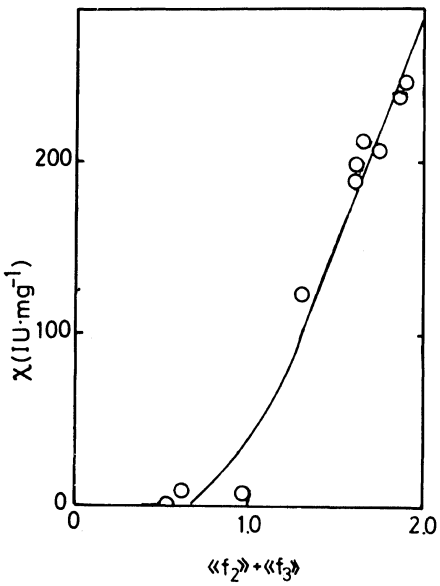

Figure 9. Effect of the sum of the substitution degree at carbon positions 2 and 3, ⟪f_2⟫+⟪f_3⟫, on anticoagulant activity χ.

predominantly governed by (⟪f_2⟫+⟪f_3⟫)/3, being larger for larger (⟪f_2⟫+⟪f_3⟫)/3 at constant ⟪f_6⟫/3 or ⟪F⟫/3. If compared at a fixed (⟪f_2⟫+⟪f_3⟫)/3, χ is larger for smaller ⟪f_6⟫/3 and ⟪F⟫/3. However, ⟪f_6⟫/3 and ⟪F⟫/3 are not very sensitive to χ. Figure 9 indicates that χ corresponding to the maximum value of (⟪f_2⟫+⟪f_3⟫) (=2) is 285.

These results seem to be the first demonstration

of a close correlation between physiological activity and chemical structure of macromolecules. To obtain a polymer drug with desirable physiological activity, it is important to make a precise design of the molecular structure. For many years, it has been known that the anticoagulant activity of naturally-occurring heparin varies significantly with its source and preparative method. The cause of this phenomenon can be understood if the distribution of chemical structure in heparin is fully elucidated, as in the case of NaCS.

Figures 10 and 11 illustrate χ and LD_{50} as functions of $\langle\langle f_2\rangle\rangle+\langle\langle f_3\rangle\rangle$ and M_n. The full lines represent the contours of the same χ (Figure 10) and LD_{50} (Figure 11). Larger $\langle\langle f_2\rangle\rangle+\langle\langle f_3\rangle\rangle$ gives larger χ when compared at the same M_n. For fixed $\langle\langle f_2\rangle\rangle+\langle\langle f_3\rangle\rangle$, χ tends to increase with a decrease in M_n. From the anticoagulant activity point of view, it is highly desirable to prepare NaCS with higher $\langle\langle f_2\rangle\rangle+\langle\langle f_3\rangle\rangle$ and lower M_n. This suggests that the mobility of NaCS in solution is a minor factor in its anticoagulant activity. In Figure 11, the contours of the same LD_{50} are convex against M_n axis, and the broken line M connecting the points for maximum

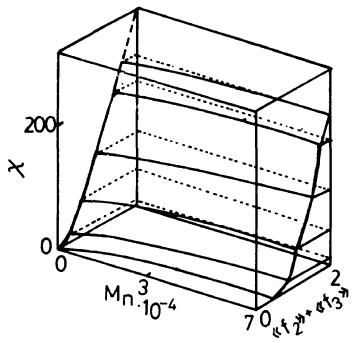

Figure 10. Anticoagulant activity χ plotted against number-average molecular weight M_n and the sum of the substitution degree at carbon positions 2 and 3, $\langle\langle f_2\rangle\rangle+\langle\langle f_3\rangle\rangle$.

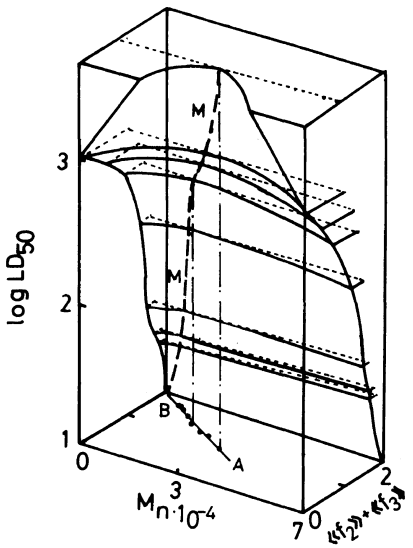

Figure 11. Plots of acute toxicity LD_{50} of NaCS against M_n and $\langle\langle f_2\rangle\rangle+\langle\langle f_3\rangle\rangle$. The line M represents maximum values of $\langle\langle f_2\rangle\rangle+\langle\langle f_3\rangle\rangle$ for a given LD_{50} (hereafter denoted by $(\langle\langle f_2\rangle\rangle+\langle\langle f_3\rangle\rangle)_m$. M_n on the line M is denoted by $(M_n)_m$. AB denotes the projection of the line M on the plane $LD_{50}=0$.

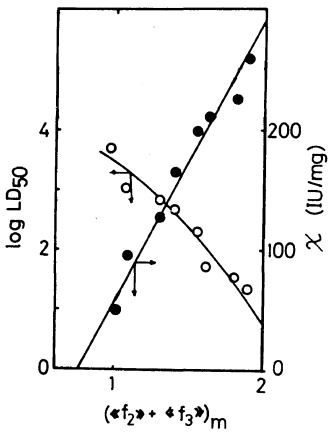

Figure 12. Relation between $(\langle\langle f_2\rangle\rangle+\langle\langle f_3\rangle\rangle)_m$ and acute toxicity LD_{50} or anticoagulant activity.

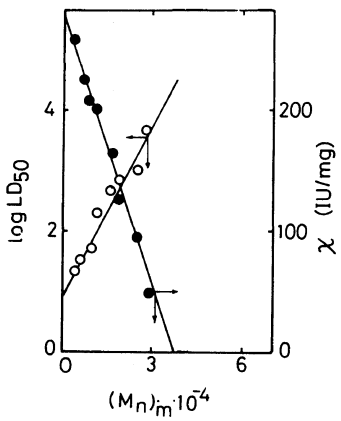

Figure 13. Relation between $(M_n)_m$ and acute toxicity LD_{50} or anticoagulant activity χ.

Table II. Existence ratios of coagulation factors and degree of inhibitory action $100(Y_0 - Y)/Y_0$ of anticoagulants

Anticoagulants	χ / IU/mg	Y / %								$100(Y_0 - Y)/Y_0$ / %							
		II	V	VII	X	VIII	IX	XI	XII	II	V	VII	X	VIII	IX	XI	XII
Heparin[a]	188—228	41.5	102.5	2.4	42.2	90.6	0.0	86.6	3.5	57.5	24.2	97.6	64.8	30.1	100	27.7	99.1
CS-12	200—222	31.6	112.2	2.2	38.9	26.3	20.8	78.4	51.5	68.4	16.9	97.8	67.5	80.0	76.0	34.6	85.6
CSD	117—130	—	112.2	2.4	34.1	63.0	24.4	103.4	157.0	—	16.9	97.6	71.5	51.9	71.9	13.7	56.0
CS-5	51—61	30.1	94.7	2.4	47.3	115.5	78.3	119.8	483.9	69.2	29.8	97.6	60.5	11.9	9.8	8.0	35.6
HBH	12—15	—	85.1	2.3	47.3	115.5	62.8	—	—	—	37.0	97.7	60.5	11.9	27.7	—	—
HBSD	10—11	—	78.7	—	—	—	—	—	—	—	41.6	—	—	—	—	—	—
CS-A	7—8	—	107.1	2.4	—	120.8	68.8	111.3	—	—	20.6	97.6	—	7.9	20.8	7.1	—
HB-1	0	44.1	89.1	2.3	50:0	—	—	—	—	54.9	34.0	97.7	57.8	—	—	—	—
None[b]	—	97.7	134.2	100	119.8	131.1	86.8	119.8	356.7	—	—	—	—	—	—	—	—

[a] Purified heparin.
[b] Denotes Y_0 for each coagulation factor in sample plasma.

$\langle\langle f_2\rangle\rangle+\langle\langle f_3\rangle\rangle$ at a given LD_{50} shifts to lower M_n with a decrease in LD_{50}, as can be seen from the projected curve AB on the plane of $LD_{50}=0$. $\langle\langle f_2\rangle\rangle+\langle\langle f_3\rangle\rangle$ and M_n at a point on the line M are denoted hereafter by $(\langle\langle f_2\rangle\rangle+\langle\langle f_3\rangle\rangle)_m$ and $(M_n)_m$, respectively. When compared at the same M_n, LD_{50} decreases as $\langle\langle f_2\rangle\rangle+\langle\langle f_3\rangle\rangle$ increases. This condition causes trouble in obtaining NaCS with a high anticoagulant activity and a desirable LD_{50}. The line M represents maximum $\langle\langle f_2\rangle\rangle+\langle\langle f_3\rangle\rangle$ for a NaCS sample, hence the maximum anticoagulant activity for a given LD_{50}. This line gives $\langle\langle f_2\rangle\rangle+\langle\langle f_3\rangle\rangle=2$ at $M_n=0$ (*i.e.*, the fully substituted glucose sulfate).

Figures 12 and 13 show the effects of $(\langle\langle f_2\rangle\rangle+\langle\langle f_3\rangle\rangle)_m$ and $(M_n)_m$ on χ and LD_{50}. From these figures, χ and LD_{50} at $\langle\langle f_2\rangle\rangle+\langle\langle f_3\rangle\rangle=2$ and at $M_n=0$ are estimated to be 305 and 8, respectively. The former is in very good agreement with the value 285 estimated from Figure 9. At $\langle\langle f_2\rangle\rangle+\langle\langle f_3\rangle\rangle=0$ and $M_n=3.7\times10^4$, LD_{50} is expected to be 10000, implying that such a NaCS sample is practically non-toxic but its A_{LW} is of the N grade. No NaCS sample having an anticoagulant activity and acute toxicity equivalent to heparin ($\chi=152$ and $LD_{50}=1200$) can be anticipated, since these figures indicate that NaCS has an acute toxicity higher than heparin which exists in the artery wall of animals and human beings. The next to the best molecular and structural parameters of NaCS as anticoagulants can be estimated from Figures 12 and 13. Given LD_{50} at the level of heparin, NaCS with $M_n=1.65\times10^4$ and $\langle\langle f_2\rangle\rangle+\langle\langle f_3\rangle\rangle=1.23$ (so that χ is 120) is acceptable. If χ is desired to be 152, $M_n=2.4\times10^4$ and $\langle\langle f_2\rangle\rangle+\langle\langle f_3\rangle\rangle=1.40$ are relevant and give $LD_{50}=320$—460. It should be noted here that the oral LD_{50} of NaCS for a rat was found to be larger than 15000 mg kg^{-1}, and hence presents no toxic problem.

The above discussion is concerned entirely with whole blood. Since 12 blood coagulation factors are known, it should be of interest to determine how and what extent NaCS acts on these factors. Table II shows the existence ratio Y (%) of each coagulation factor and the anticoagulating ability $100(Y_0-Y)/Y_0$, as well as χ of the anticoagulants. Figure 14 shows $100(Y_0-Y)/Y_0$ for various coagulation factors plotted against χ. From this figure, the coagulation factors may be classified into two categories:

(1) $100(Y_0-Y)/Y_0$ varies with χ: Factors V, VIII, IX, XI, and XII. Factors VIII, XI, and XII increase in linear proportion to χ and factor IX increases rapidly with χ, approaching some asymptotic value. In contrast, factor V has a tendency to decrease gradually with increasing χ.

(2) $100(Y_0-Y)/Y_0$ is almost independent of χ and decreases in the following order: factor VII > factor X > factor II.

We cannot explain why some factors are independent of χ and others decrease with χ and, in

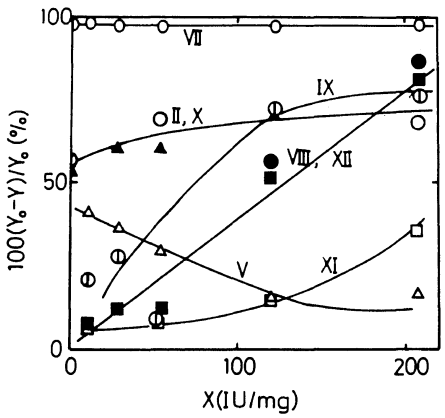

Figure 14. Correlation between $100(Y_0-Y)/Y_0$ of various coagulation factors and anticoagulant activity of NaCS: Factor II, ○; V, △; VII, ○; VIII, ■; IX, ⊙; X, ▲; XI, □; XII, ●.

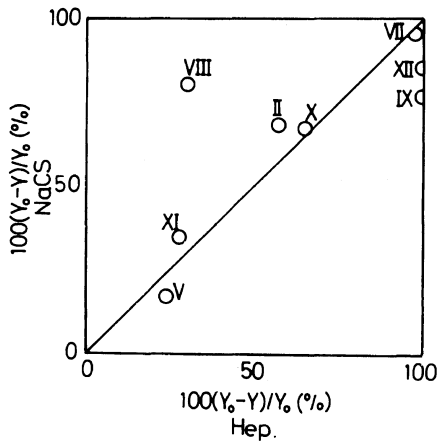

Figure 15. Comparison of $100(Y_0-Y)/Y_0$ between NaCS (CS-12) and heparin: Roman numbers indicate the order of coagulation factors. Symbols are the same as those in Figure 14.

particular, why factor VII vanished by addition of NaCS, even if the polymer has no anticoagulant activity ($\chi=0$), as in the case of sample HB-1.

The inhibitory action of NaCS is compared with that of heparin in Figure 15, where $100(Y_0-Y)/Y_0$ for the two polymers having almost the same χ value (190 ± 10) are plotted on the horizontal and vertical axes. Except for factor VIII, the action of NaCS on all coagulation factors is the same as that of heparin. NaCS inhibits the action of factor VIII (antithermophilic globulin) much more effectively than heparin. Therefore, it may be concluded that NaCS acts mainly on factor VIII and partly on factor IX (plasma thromboplastin component); the latter was found to be suppressed by heparin.[19]

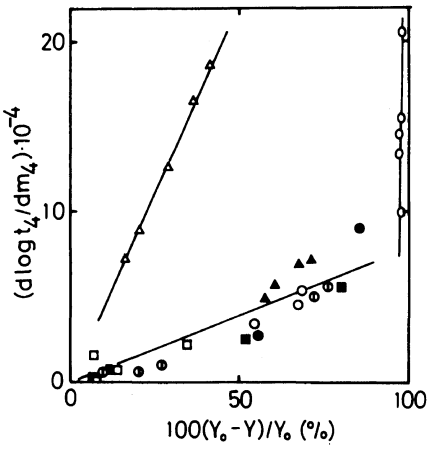

Figure 16. Plots of $d\log t_4/dm_4$ against $100(Y_0-Y)/Y_0$ for various coagulation factors. Symbols are the same as those in Figure 14.

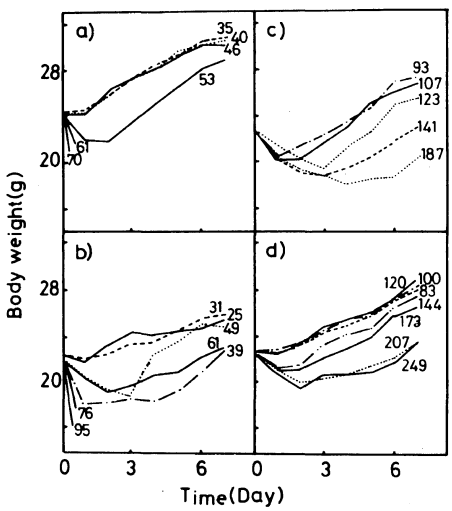

Figure 17. Changes in body-weight of rats with time following injection of a NaCS solution: a) Sample CS-11, b) Sample CS-4, c) Sample CS-12, and d) Sample CS-6. Numbers on the curves indicate the injected amounts of NaCS in $mg\,kg^{-1}$.

Figure 16 illustrates the relation between $d\log t_4/dm_4$ and $100(Y_0-Y)/Y_0$. Except for factor VII, a straight line passing the origin is obtained, although factor V is expected to depend more strongly on concentration than the other factors.

Figures 17(a)—(d) illustrate time changes in the body-weight of rats after saline solutions of NaCS samples with four different LD_{50} (43, 53.6, 63.8 and 184.5) were injected. In obtaining these data, we prepared polymer solutions of different concentrations and injected the same volume of each solution into a rat. The numbers on the lines denote the injected NaCS expressed in $mg\,kg^{-1}$. For all NaCS samples, when the dose amount was less than the LD_{50} of a given NaCS, there was progressive

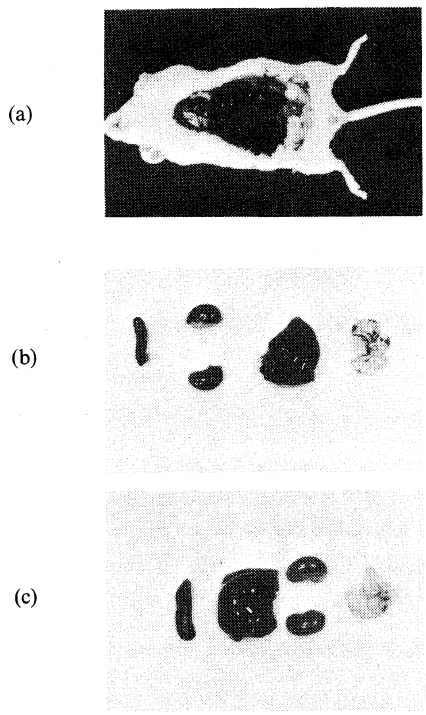

Figure 18. Typical anotomical photographs of live rats (a) and those of the intestinal organs of live (b) and dead (c) rats.

body-weight increase. When the dose amount was more than the LD_{50}, body-weight decreased at least initially, passed a minimum, and then increased monotonically. If the body-weight loss ratio (B_{WL}) is defined as the maximum body-weight loss M (g) divided by the dose amount m ($mg\,kg^{-1}$) and the LD_{50} of a given NaCS, that is, $B_{WL} = M/m \cdot LD_{50}$, then B_{WL} for all samples comes close to 0.08. This indicates that body-weight loss in rats is determined primarily by the dose amount and the LD_{50} of the NaCS sample used.

General symptoms of rats after injection of NaCS were occasional staggering motion and depression of breathing. Some rats lay down and often fell into a fit of convulsions. However, these symptoms disappeared one to four days after the injection. The dead rats showed bleeding from nose and mouth. The bleeding seemed greater for larger volumes of injected NaCS solution. In live rats, anemia was found.

Figure 18 shows some typical anatomical photographs of live rats (a) and the intestinal organs of live (b) and dead (c) rats after injection of NaCS. In the dead rats, the liver showed anemia and the lung, congestion and bloodshots. These abnormal symptoms were conspicuous in rats to which larger amounts of NaCS had been administered. In the live rats, no anormalous symptoms were found.

It is generally known that muco-polysaccharide derivatives including heparin exhibit a wide variety of pharmaceutical activities. In this work, we have shown that the most important factor governing the anticoagulant activity of NaCS is not the average molecular weight but the average chemical structure. It would be fruitful to carry out further research on the yet poorly understood physiological activities of polymer drugs by the methods presented in this study.

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